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DNA INCORPORATION AND NUCLEAR DIVISION IN SYNCHRONOUS MITOTIC CULTURES OF *USTILAGO HORDEI* (PERS.) LAGERH.

by

PAUL UNRAU

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF MASTER OF SCIENCE

DEPARTMENT OF GENETICS

EDMONTON, ALBERTA

AUGUST, 1965

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "DNA Incorporation and Nuclear Division in Synchronous Mitotic Cultures of *Ustilago hordei* (Pers.) Lagerh." submitted by Paul Unrau in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

The mitotic cycle of synchronously-dividing haploid sporidia of Ustilago hordei (Pers.) Lagerh. was studied by means of DNA-extraction and cytological procedures. Sporidia were stained utilizing the Feulgen technique and nuclear structure was correlated to the relative quantities of DNA present in the cells. The time of nuclear incorporation of DNA was thus determined.

It was found that the amount of DNA increased immediately after completion of the first nuclear division, leading to the supposition that the normal DNA content of the "resting" nucleus is twice the amount that would normally be expected of the haploid nucleus.

ACKNOWLEDGEMENTS

The writer expresses his sincere thanks to Dr. Clayton Person, Department of Genetics, University of Alberta, for his advice and assistance during the investigation and particularly the preparation of this manuscript.

Thanks are also expressed to C. H. Hood for advice and aid particularly in matters of materials, synchronization procedures, and discussion of the results. Dr. E. M. Pearce who provided instruction in the Schmidt-Tannhauser DNA extraction method as well as greater respect for precise technique, the Division of Plant Biochemistry who lent their sonifier, and the National Research Council which provided a bursary are also gratefully acknowledged.

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INTRODUCTION

Several stages in the life cycle of species of Ustilago have been used advantageously in genetic studies. Kniep (12) recognized as early as 1919 the bipolar sexual mating mechanism shown by sporidia. Hüttig (11) recognized that bridge formation between promycelial cells of the germinating teliospore of U. avenae could be used to measure the occurrence of crossing-over involving the mating locus. Nicolaisen (16) and others have utilized the interactions of the host with various sporidial combinations to determine the genetics of virulence and resistance. Holliday (7) and Esposito and Holliday (5) used synchronized cultures of different strains of vegetative, heterozygous diploid cells to gain new information relating to mitotic crossing-over and mitotic cell division.

Holliday (7) found that DNA replication in U. maydis had taken place before the induction of the first synchronized cell division; no DNA synthesis took place during the first synchronized division. DNA synthesis occurred immediately following completion of the first nuclear division. He therefore concluded that the vegetative nuclei of these diploid cells have twice the content of DNA expected of a diploid nucleus. Williamson and Scopes (25) found that in yeast synchronized by a similar procedure DNA replication immediately preceded the first division. Weijer and Koopmans (23) obtained essentially the same result in conidial cells of Neurospora crassa. The present experiments, carried out with a different Ustilago species, provided the opportunity to extend Holliday's results.

In the present experiment, synchronous cultures of U. hordei were studied with a view to determining the relationships of cell division, nuclear division, and DNA replication. Campbell (3) denotes synchronization as "any process in which the events in different cell lines are put into phase with each other." The induction of synchrony may be achieved following either of two procedures:

(i) prior treatment, in which cultures receive a single, prior treatment and are then allowed to grow unrestrictedly; and

(ii) periodic treatment, in which a succession of environmental changes brings the cells into synchrony.

Experiments of the former type include the work of Newton and Wildy (15) with HeLa cells as well as the work presented here, while examples of the latter include the work of Scherbaum (17) with Tetrahymena pyriformis, of Williamson and Scopes (26) with yeast, and of Holliday (7) with U. maydis.

A closer definition of the degree of synchronization involves the calculation of the relative numbers of cells undergoing any particular process at any particular time. The "synchronization index" employed by Williamson and Scopes (26) is a measure of the relative degree of synchrony. Calculation involves cell counts before and after division as well as an accurate measure of the period of cell division and the duration of a generation. The formula $\left(\frac{n}{n_o} - 1\right)\left(1 - \frac{t}{g}\right)$ (where n is the

post division cell number, n_o the original cell number, t the time required for nuclear division and g the time required to complete one generation) provides a relative value for the degree of synchrony of any culture.

The terminology relating to DNA-content of dividing cells requires some clarification for the purposes of this study. Since the analytical technique used excluded measurement of free DNA nucleotides, only those nucleotides were measured that had been incorporated as polynucleotides, presumably into chromosomes. The measured increases in DNA content will therefore be referred to as DNA incorporation rather than as DNA replication or DNA synthesis.

MATERIALS AND METHODS

Stocks

The haploid, sporidial cultures of *U. hordei* used in this experiment were all derived from single sporidia that had been isolated from germinating teliospores. The cultures were routinely maintained at 22° C in shake culture and were transferred to fresh medium at 48-hour intervals when cell concentrations were about 2.5×10^8 cells per ml.

Medium

Vogel's (24) complete medium was used throughout. This medium contains 5% hydrolysed casein and 5% yeast extract, in addition to sucrose and the specified mineral salts.

Synchronization

The procedure for obtaining synchronization of mitosis in sporidial cultures of *U. hordei* suggested by C.H. Hood (9) were followed. Haploid cultures were incubated 48 hours in shake culture and then transferred to fresh Vogel's medium. The synchronized divisions occur immediately on transfer to fresh medium. Samples taken at half-hour intervals provided material for cell counts and photomicrography.

DNA extraction

DNA was extracted according to a simplified technique described by Esposito and Holliday (5). The method used is outlined in the flow sheet (Fig. 1) and is similar to the method cited except that the cells were suspended in a 2:1 methanol:chloroform mixture. Although this was done to kill the cells as rapidly as possible it also served to extract the alcohol-soluble proteins. Berton (2) states that the diphenylamine reaction is specific for deoxyribose sugars and this reaction is used throughout to measure DNA incorporation.

Staining

At the same time that 50 ml samples were removed for centrifugation and extraction, 10 ml samples were also removed for cytological study. The cells were killed and fixed in Carnoy's solution* (1) and after 30 minutes the fixative was decanted and the cells were stored in 70% ethanol in the freezer.

The cells were dried onto coverslips, hydrolysed for 9 minutes in 1 N HCl at 60° C, and stained for one-half to three-quarters of an hour in Feulgen reagent. They were then rinsed in tap-water for 5 minutes, dehydrated in absolute alcohol and, after removal by flotation, the coverslips were replaced using a drop of Permount.

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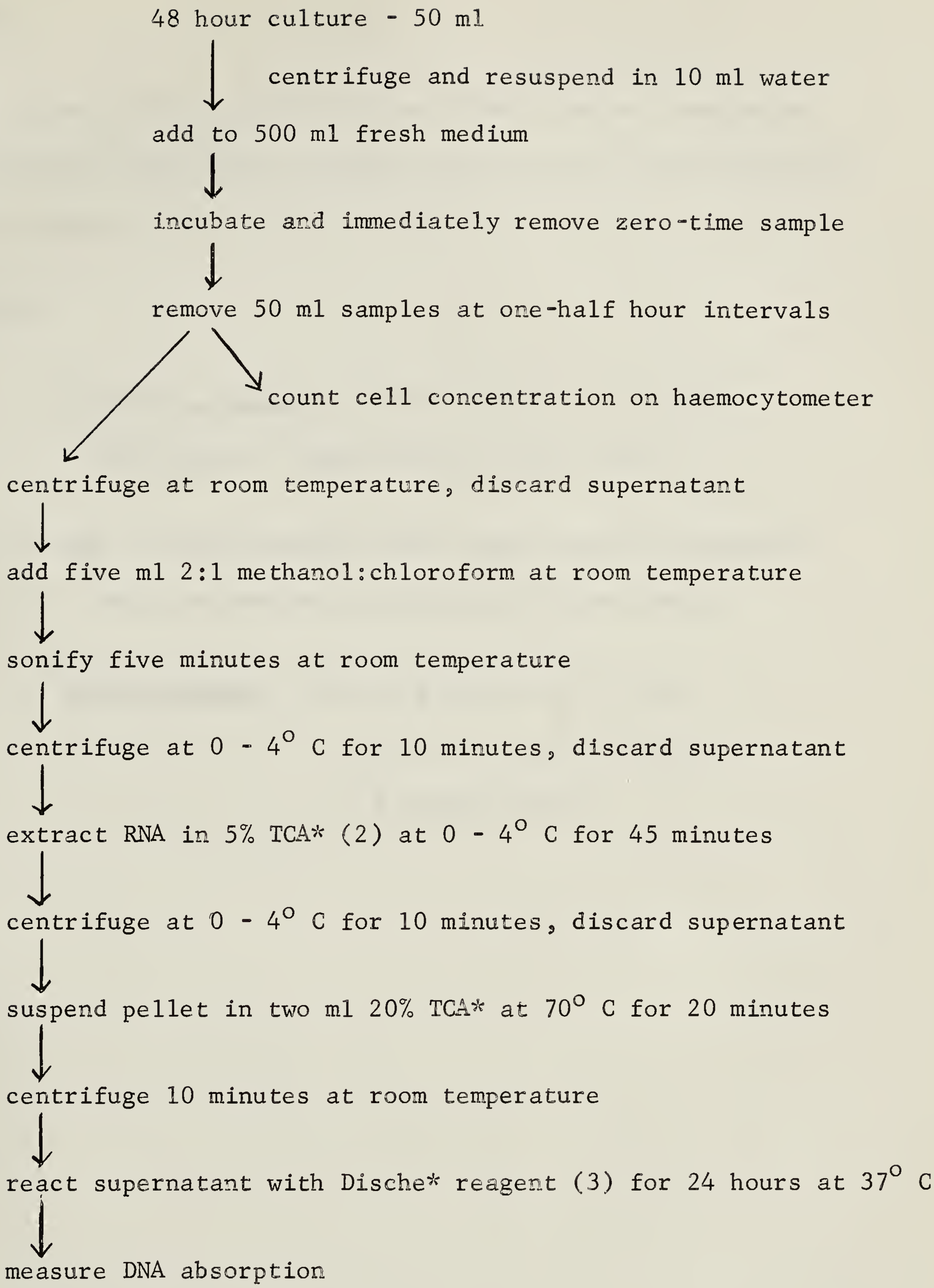
* For formulations see page 7.



48 hour culture - 50 ml

centrifuge and resuspend in 10 ml water

add to 500 ml fresh medium

incubate and immediately remove zero-time sample

remove 50 ml samples at one-half hour intervals

count cell concentration on haemocytometer

centrifuge at room temperature, discard supernatant

add five ml 2:1 methanol:chloroform at room temperature

sonify five minutes at room temperature

centrifuge at 0 - 4° C for 10 minutes, discard supernatant

extract RNA in 5% TCA* (2) at 0 - 4° C for 45 minutes

centrifuge at 0 - 4° C for 10 minutes, discard supernatant

suspend pellet in two ml 20% TCA* at 70° C for 20 minutes

centrifuge 10 minutes at room temperature

react supernatant with Dische* reagent (3) for 24 hours at 37° C

measure DNA absorption

Fig. 1. DNA extraction flow sheet

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* For formulations see page 7.

Photography

Representative fields were selected under oil immersion and photographed using Kodak contrast-process Ortho film and printed on Kodak F4 paper.

Formulations

1. Carnoy's solution - 6 parts ethanol : 3 parts chloroform : 1 part glacial acetic acid.

2. TCA - Trichloroacetic acid; the 5 and 20% solutions were prepared on a weight per volume basis.

3. Dische reagent - 100 ml glacial acetic acid
   2.75 ml concentrated H_2SO_4
   1 g diphenylamine.

RESULTS

DNA extractions and cell counts

The results of DNA extraction and cell counts are shown in Figures 2 to 4.

Figure 2 shows the results of DNA extraction using a modification of the Schneider-Schmidt-Tannhauser DNA extraction technique described by McLeish (14). The procedure was abandoned after this result because of its excessive length and complexity, as well as its failure to provide consistent results. After adopting the method suggested by Esposito and Holliday (5) the results shown in Figures 3 and 4 were obtained. It will be noticed that in all experiments the increase in DNA incorporation precedes the increase in cell numbers, and that DNA levels remain relatively constant during most of the interval in which cell numbers are increasing. However, the generation time varied from one experiment to another. For the experiment described in Figure 4 the generation time is estimated at 90 minutes, whereas in the other two experiments (Figures 2 and 3) the estimated generation time is 120 minutes.

Cytology

At the time of transfer to new medium the sporidial cells are uniform in shape and size, and the nuclei appear diffuse (Plate 1).

Following transfer, the cells at 60 minutes have begun to form buds, and the nuclei have begun to appear more clearly

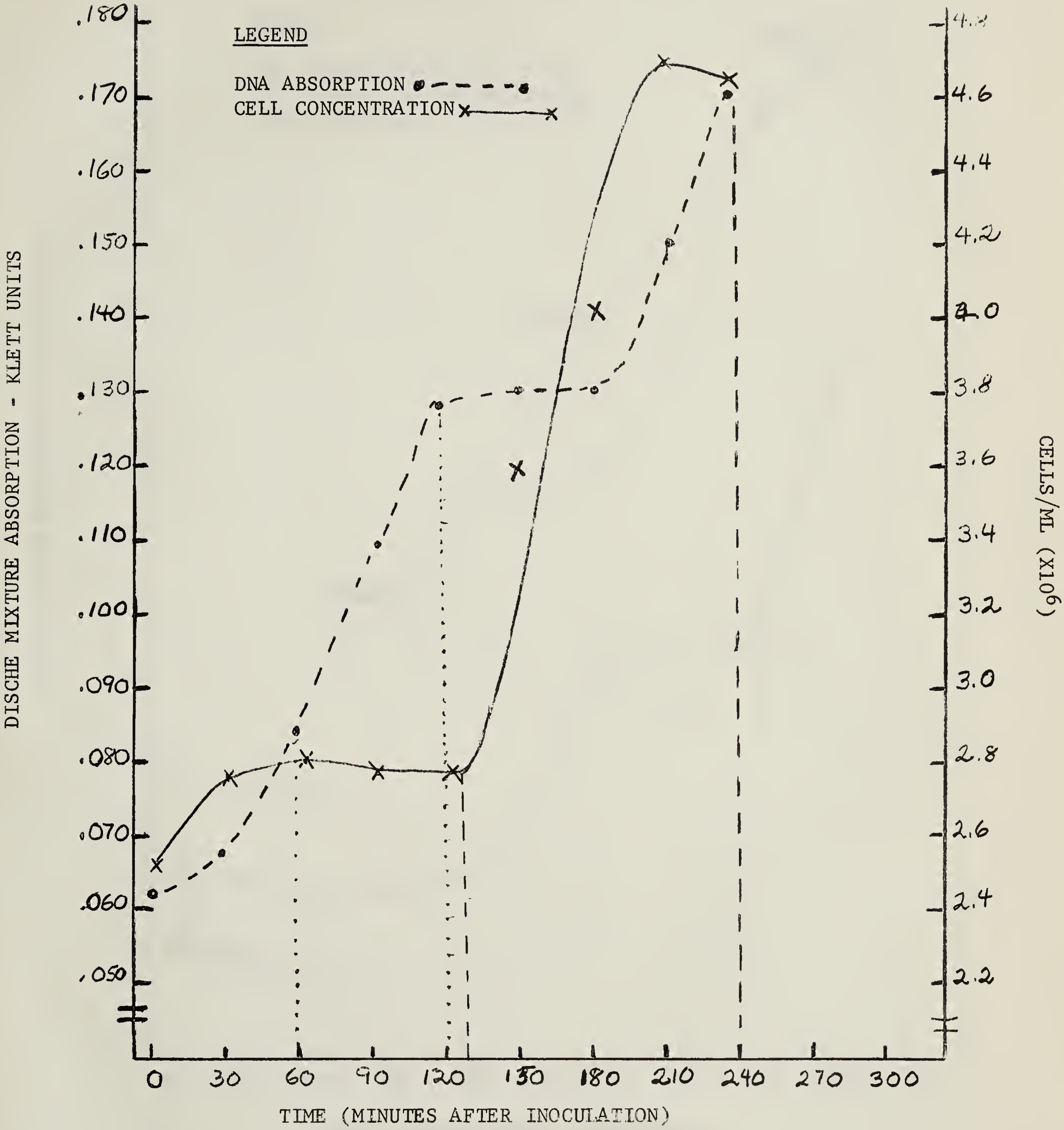


Fig. 2. Correlation of DNA replication and cell increase, McLeish (14) extraction.
Synchronization index = .52
Generation time = 120 min; Division time = 35 min.

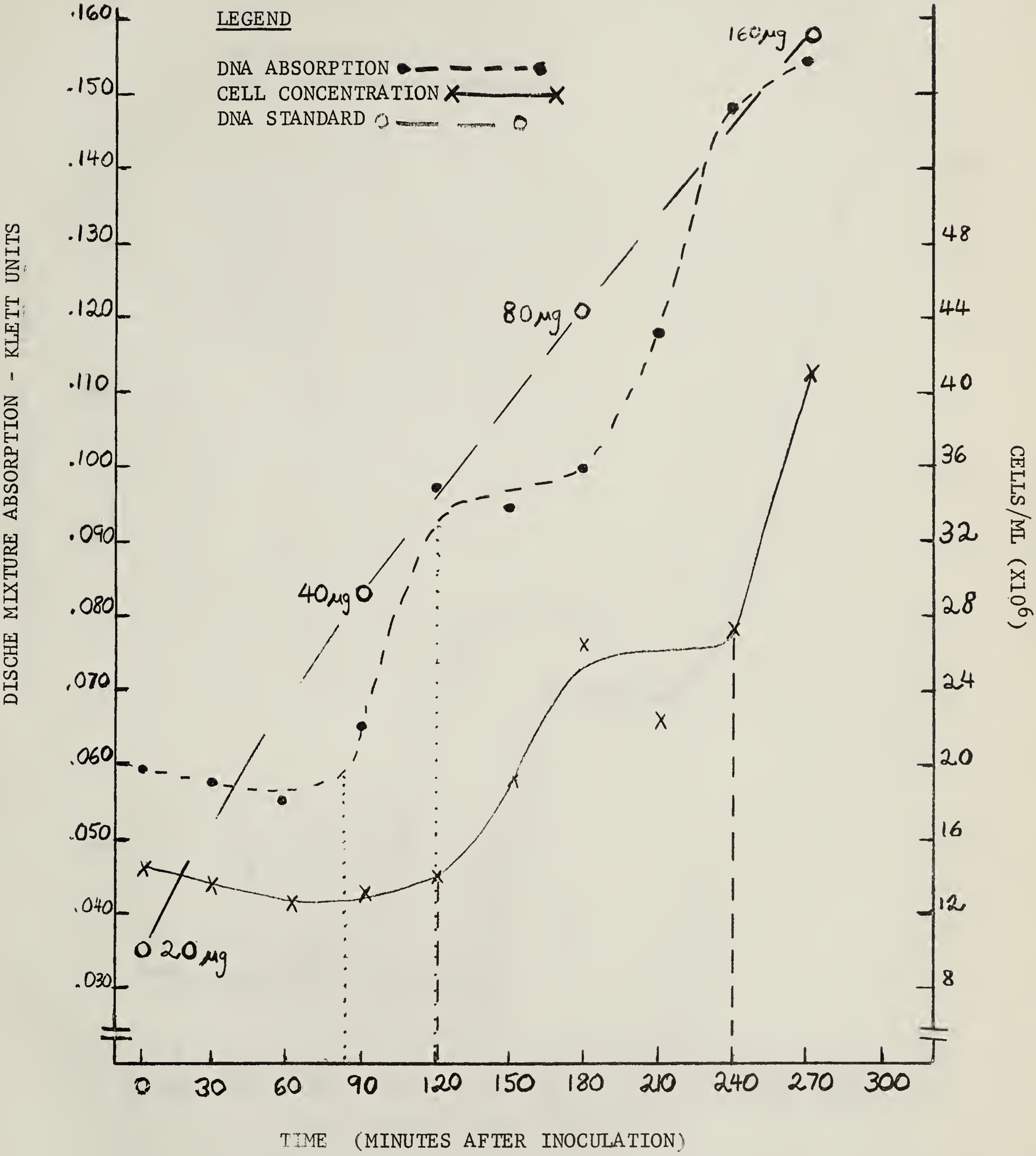


Fig. 3. Correlation of DNA replication, cell number, and DNA concentration.
Synchronization index = .50
Generation time = 120 min; Division time = 35 min.

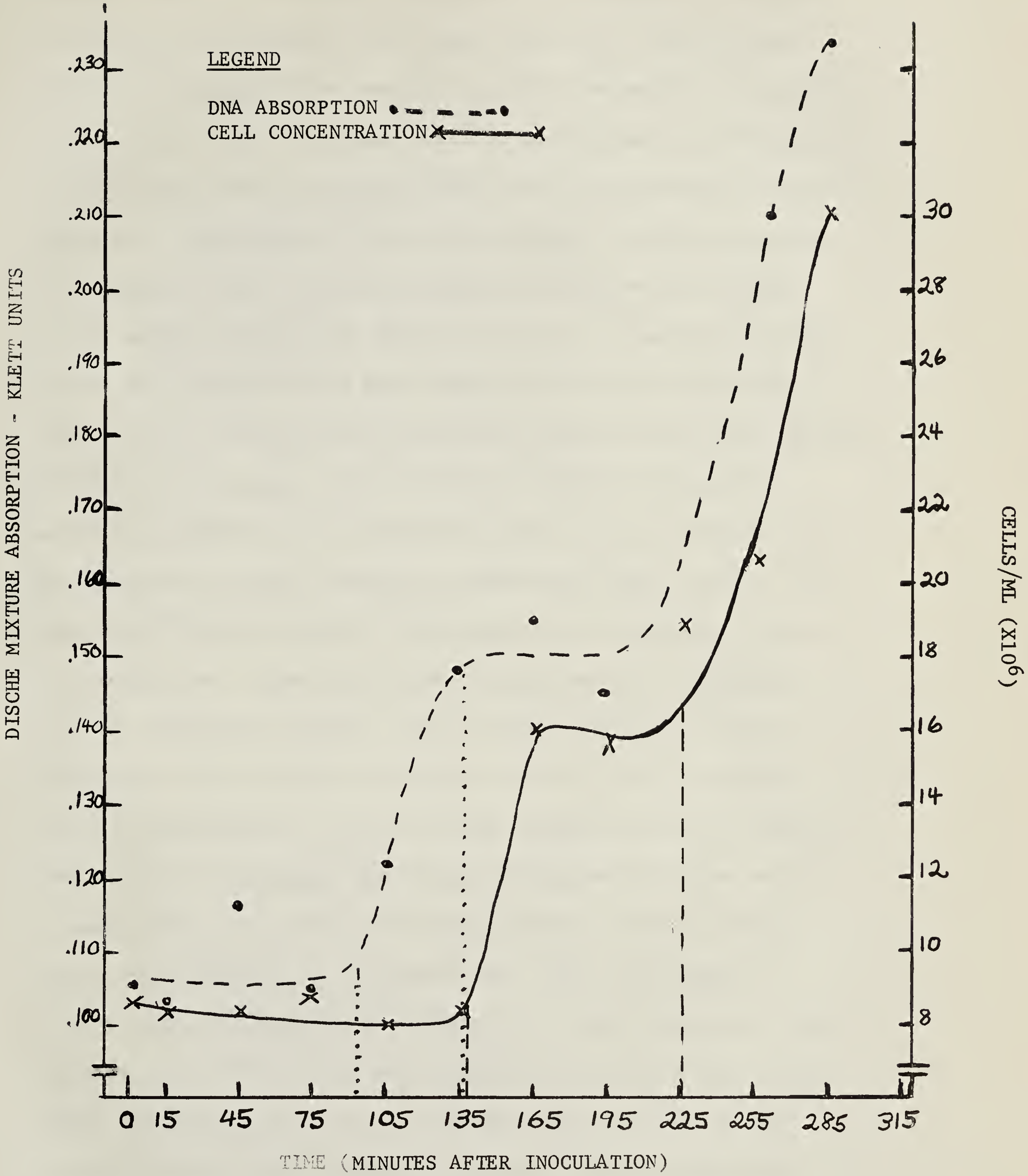


Fig. 4. Correlation of DNA replication and cell number.
Synchronization index = .66
Generation time = 90 min; Division time = 35 min.

defined (Plate 2). Further condensation of the nuclei is apparent by 80 minutes and the buds are larger (Plate 3). Plate 4 shows cells at 90 minutes after transfer in which the nuclei, now clearly defined, have begun to migrate into the growing buds. At this time the buds have reached about two-thirds their ultimate size, and the nuclei that have migrated into them are about to divide. A group of 90-minute cells, showing the sequence of events that occurs during nuclear division, is shown in Plate 5. It may be seen that one of the daughter nuclei moves from the bud back to the parent cell. Cells photographed at 105 minutes (Plate 6) show that nuclear division is continuing. At 120 minutes (Plate 7) it is still possible to identify a few nuclei that have recently divided, but the process of nuclear division is effectively over. However the buds, which remain attached to the parent cell, continue to grow to maximum size. Separation occurs during the 120 - 180 minute interval following transfer. Plate 8 shows a group of cells at 150 minutes, one of which is forming a new bud. At 180 minutes (Plate 9) separation of the fully-grown daughter-cells is complete. Some cells at 180 minutes have begun to form new buds, and nuclei in many cells at this time have become clearly defined. These nuclei are considered to be pre-mitotic. The cells shown in Plate 10 were photographed at 210 minutes. Cells observed at this time are not uniform. Some are undergoing nuclear division while others are forming buds. It is inferred that the cells present at this time fall into parental and daughter classes, and that division is proceeding more rapidly in the newly-formed daughter cells.

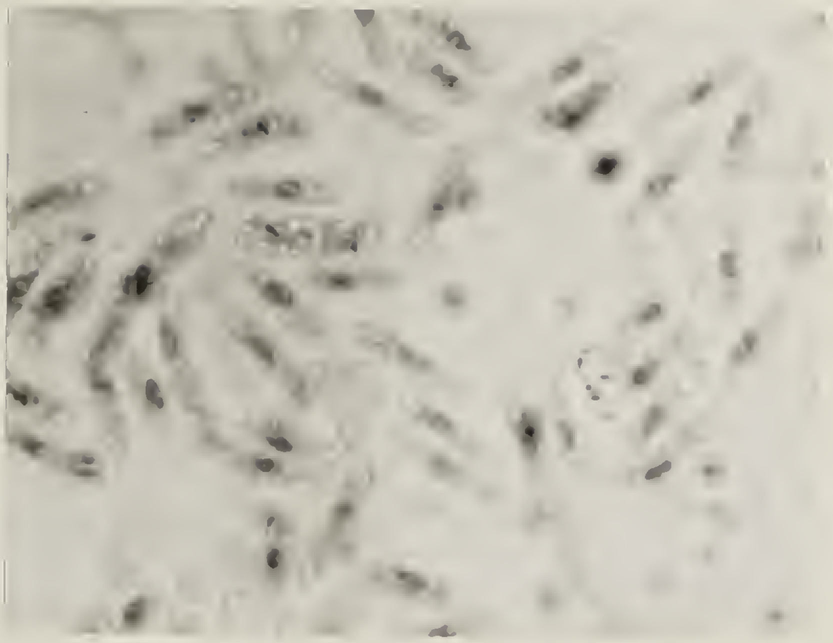


Plate 1. 0 time sample showing synchronized inoculum immediately after inoculation.

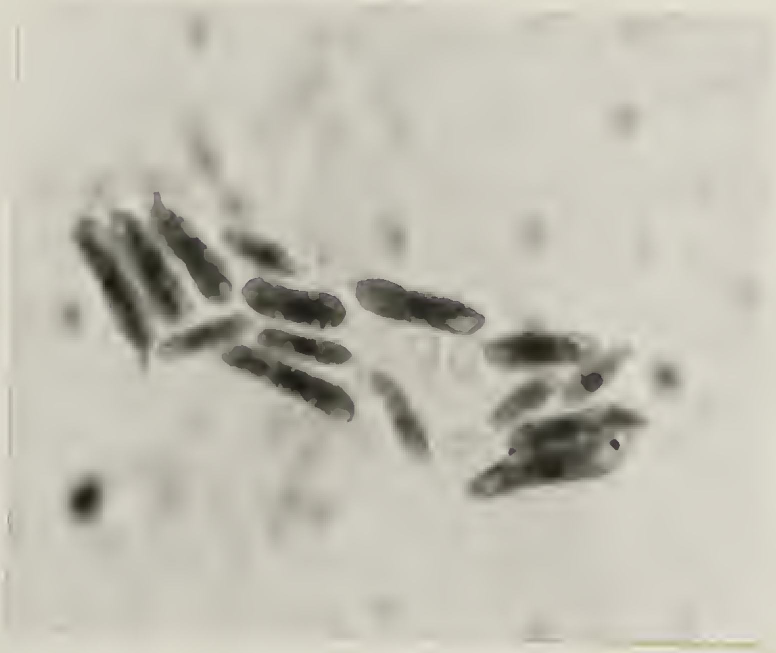
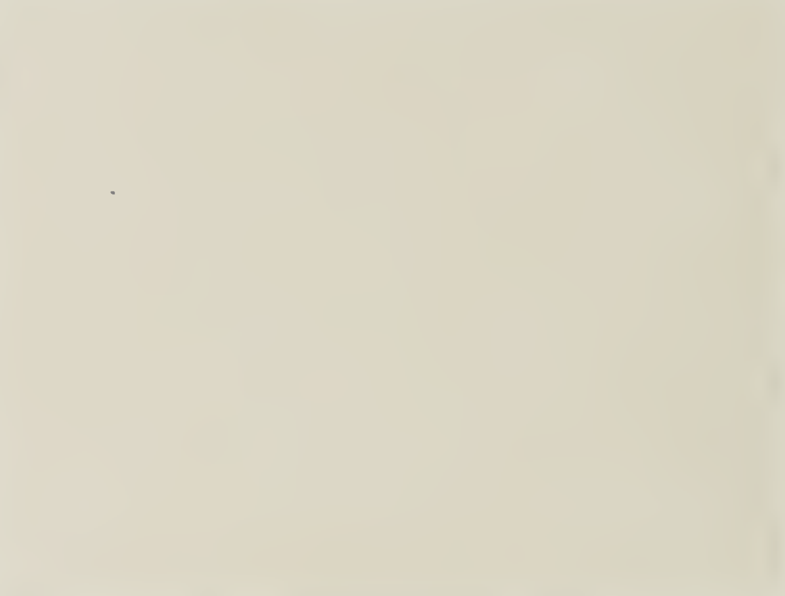


Plate 2. 60 minute sample.



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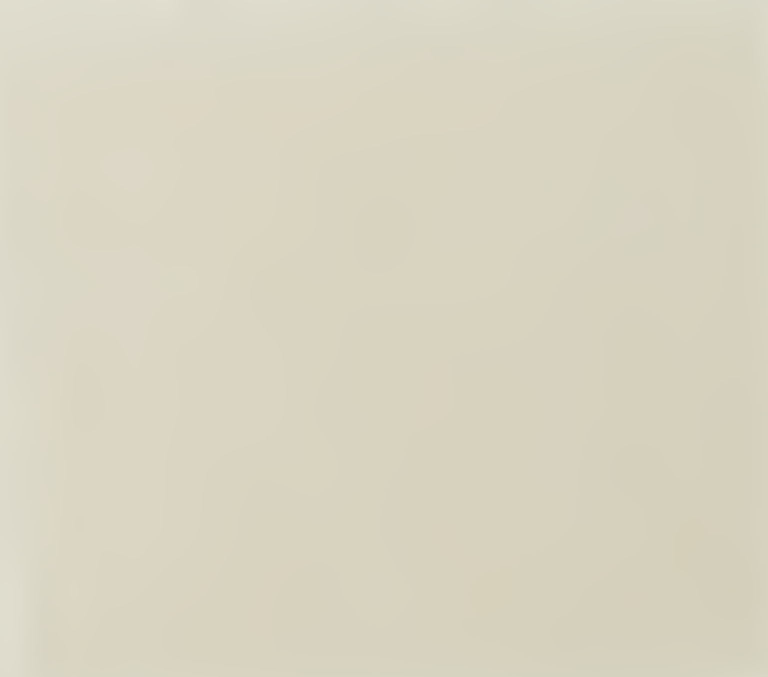

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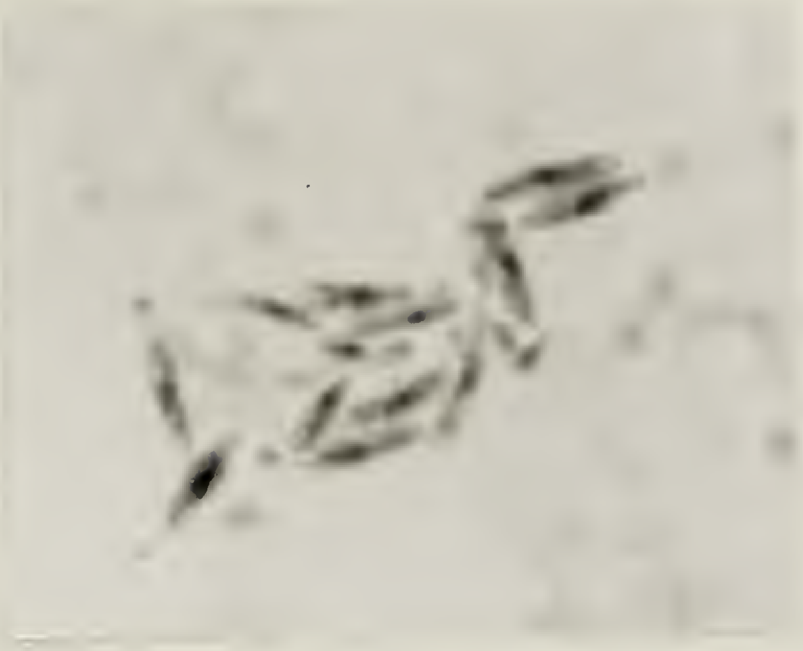

Plate 3. 80 minute sample.

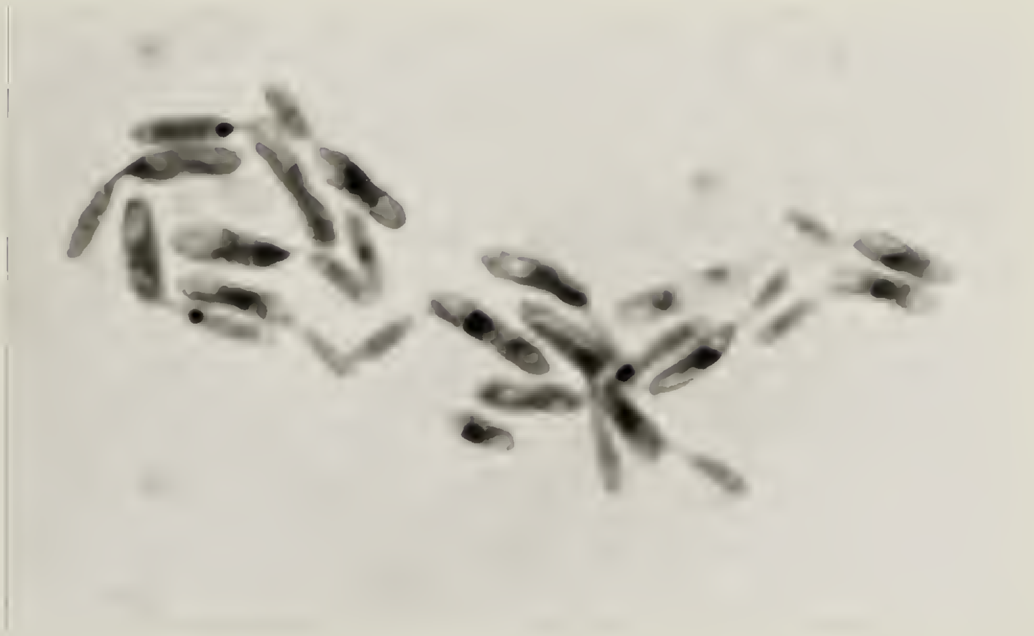

Plate 4. 90 minute sample showing premitotic and mitotic nuclei.

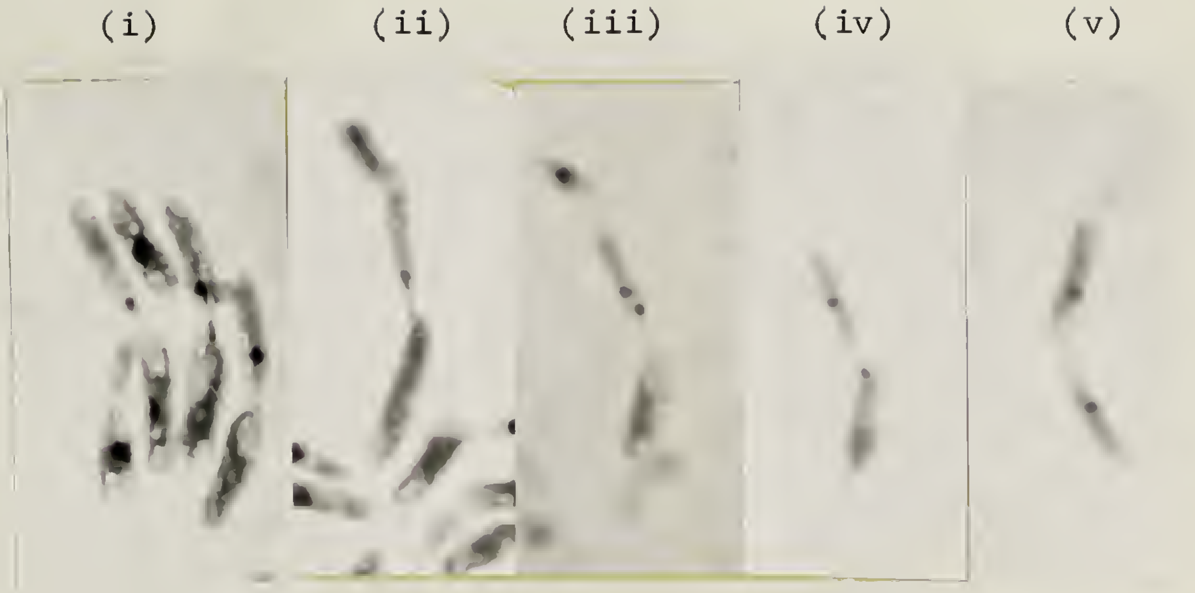

Plate 5. Types of cells found at 90 minutes showing (i) premitotic nucleus, (ii) nucleus entering young sporidium, (iii) nucleus dividing in young sporidium, (iv) one daughter nucleus re-entering old sporidium, (v) completed nuclear division.

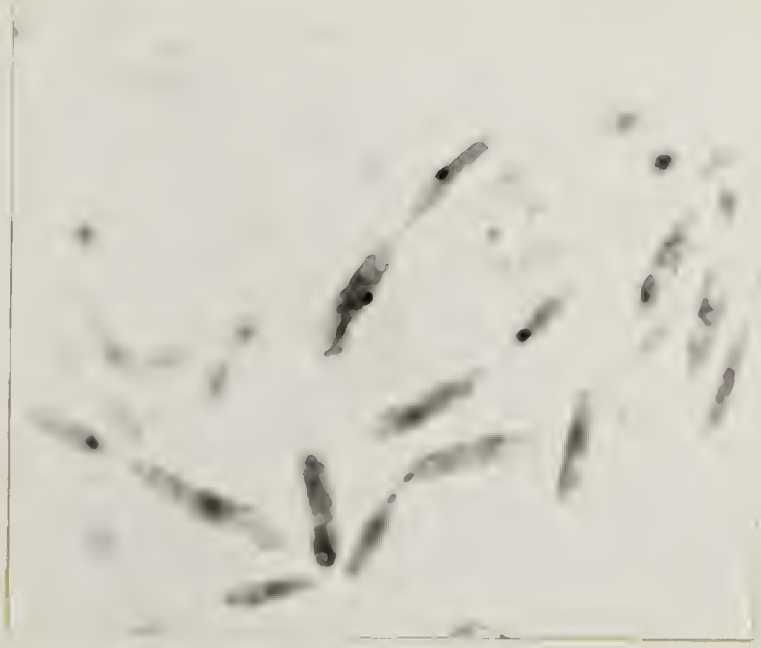

Plate 6. 105 minute sample showing mitotic cells.

Plate 7. 120 minute sample showing a field of post-mitotic cells.

Plate 8. 150 minute sample showing some divided cells.

Plate 9. 180 minute sample showing complete division and resumption of division cycle.

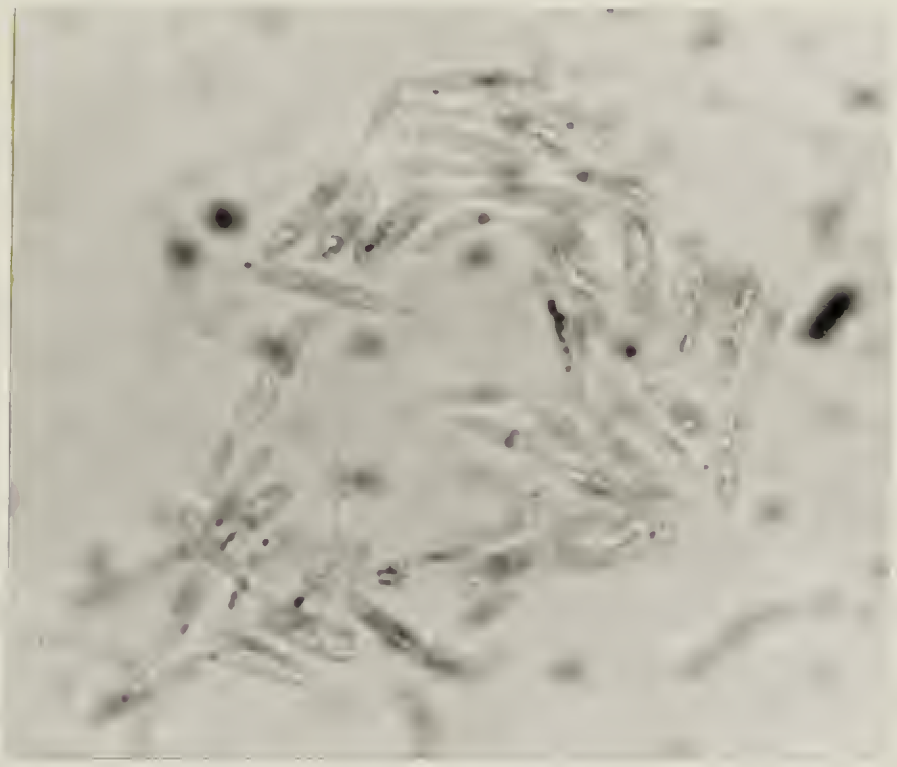

Plate 10. 210 minute sample with division in young and old sporidia proceeding at different rates.

DISCUSSION

When the sequences of events as determined by DNA-extraction and cell counts (Figs. 2 - 4) and by cytological examination (Plates 1 - 10) are brought together the interrelationships between them become evident. It is apparent that nuclear division has been completed in the majority of cells by 120 minutes (Plate 7). It is also apparent (Figs. 2 and 3) that DNA incorporation has taken place by 120 minutes. Although there is little reason to expect DNA incorporation and nuclear division to occur simultaneously, since this has never been reported, this possibility will be referred to later. Based on the information presented in Figure 4, where DNA incorporation is not completed until 135 minutes after transfer, it is considered more likely that DNA is incorporated immediately after nuclear division. This interpretation is in agreement with Holliday's (7) conclusions.

Bever (1) showed in hybridization experiments that different species of _Ustilago_ show in many ways their common ancestry. The agreement between analytical results obtained by Holliday with _U. maydis_ (7) and results obtained in the present study with _U. hordei_ may also be taken as evidence of close genetic affinity.

Unpublished work of Hood (9) shows that synchronized cultures of _U. hordei_ are UV-sensitive, showing increased mutability at 90 minutes and again at 150 minutes after transfer to new medium.

The peak at 90 minutes coincides with the beginning of nuclear division as determined in this study, and the second peak, at 150 minutes occurs during a period when nuclear division is not in progress. This may be taken to indicate that there are two different points in the life cycle that are sensitive to UV-treatment.

On the basis of work by many investigators it appears that within the mitotic cycle there is no standard time for either DNA synthesis or chromosomal replication. Although both processes are reported to occur during interphase, there is no general rule that applies to all organisms (Mazia, 13). For example, Newton and Wildy (15), who worked with parasynchronous HeLa cultures, found that DNA synthesis began shortly after telophase and continued throughout interphase, whereas chromosomal replication occurred at the termination of interphase. Taylor (21), who correlated DNA replication with a period in which the chromosomes of *Tulbaghia* are most prone to x-ray breakage, concluded that chromosomal replication in *Tulbaghia* occurs during early interphase. Taylor (20) had previously noted that DNA synthesis may occur during early, middle or late interphase. Weijer (24) observed that increased radiosensitivity in synchronous conidial cultures of *N. crassa* occurred during the time of duplication of genetic material. Howard and Pelc (10) found DNA replication in *Vicia faba* to take place in middle interphase.

If DNA replicates in *U. hordei* immediately after nuclear division, the resulting nuclei must contain either the C/2, or C,

or some multiple of the C amount of DNA. By convention, the amount of DNA present in a haploid cell is referred to as the C amount. Although it is possible that daughter nuclei may contain half-helices of DNA during a brief period before replication, this possibility is considered to be remote. A much more likely interpretation is that the premitotic nucleus is 2C, and that each daughter nucleus receives the C-amount of DNA. On this interpretation the DNA content of daughter nuclei is quickly raised to the 2C level. If DNA is present at some higher multiple of C it then becomes difficult to interpret the mutation data, in which no instance of either a double mutant or a mosaic colony has been observed.

It should be possible, using techniques that distinguish between single- and double-stranded DNA, to test whether newly-formed daughter nuclei receive half-helices of DNA, as expected if premitotic nuclei contain the C-amount of DNA. Examination of whole chromosomes has been carried out by Du Praw (5), using embryonic cells of the honeybee. If it should prove possible to apply this technique to whole chromosomes of U. hordei, new information relating to the number of DNA helices present in pre- and postmitotic nuclei may be gained.

The system employed in this study could also be used for other types of analysis. For some genes it should be possible to determine the time of replication and, for others, the time of gene action. Accumulation of specific m-RNA fractions at different times during the cell cycle, using methods such as puromycin blockage,

may lead to the recognition of genetic activity at specific loci. Studzinsky (19) found, following treatment of HeLa cells with puromycin, that RNA bodies resembling nucleoli accumulated in the cytoplasm; isolation and use of this RNA as an *in vitro* primer of protein synthesis would permit recognition of the primary gene product. Holley *et al*. (6) have determined the nucleotide sequence of a specific transfer RNA; a similar approach applied to the accumulated RNA products of specifically inhibited genes (that is to say, genes inhibited at specific points during the life cycle) may lead to the determination of nucleotide sequences in m-RNA. These could be correlated with amino-acid sequences of polypeptides formed during *in vitro* synthesis. It is difficult at the moment to estimate either the advantages or the disadvantages of using synchronized *U. hordei* cultures in studies of this nature.

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B29840